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IgM phosphorylcholine antibodies inhibit cell death and constitute a strong protection marker for atherosclerosis development, particularly in combination with other auto-antibodies against modified LDL

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ABSTRACT

Background: We have reported that anti-phosphorylcholine (anti-PC) IgM is a protection marker for human cardiovascular disease (CVD) and atherosclerosis. We here investigate the anti-PC autoantibodies in a well-defined cohort with regard to idiotype, atherosclerosis progression and mechanisms for its protective action.

Methods: Serum levels and binding specificities of different anti-PC isotypes were determined in 226 hypertensive individuals enrolled in European Lacidipine Study on Atherosclerosis using ELISA. The mean of the maximum Intima-Media Thicknesses (IMT) in the far walls of common carotids and bifurcations was assessed at the time of inclusion, and four years afterwards. Apoptosis in immune cells was induced with lysophosphatidylcholine (LPC) and quantified using the MTT-assay.

Results: Anti-PC IgM, IgA and IgG1 (but not IgG2) was negatively associated with IMT-progression. Combining anti-PC IgM with data on antibodies against oxidized- and malondialdehyde-modified LDL further strengthened this association. At very high levels, anti-PC IgM exhibited a striking negative association with atherosclerosis progression (OR 0.05; CI 0.006–0.40). Analysis of serum samples taken four years apart in study participants affirmed the stability of anti-PC IgM titers over time. Examination of fine specificities revealed that the protective isotypes (IgM, IgA and IgG1) are of the Group I idiotype whereas the non-protective IgG2 subclass was Group II. Anti-PC IgM inhibited LPC-induced cell death of immune cells.

Conclusion: Group I anti-PC antibodies, particularly of the IgM class, are independent protection markers for atherosclerosis progression. One potential mechanism of action is inhibition of LPC-induced cell cytotoxicity.

1. Introduction

Atherosclerosis, the major underlying cause of cardiovascular disease (CVD), is a chronic low grade inflammation in the artery wall [1] characterized by the accumulation of modified lipoproteins, dead cells and an abundance of activated immune cells that produce pro-inflammatory cytokines [2].

Modified forms of low density lipoprotein (LDL), such as malondialdehyde modified LDL (MDA-LDL) and oxidized LDL (oxLDL) have been linked to vascular inflammation and atherosclerosis in numerous publications utilizing a wide range of research methodologies including [3–5].

Many of the biological effects of oxLDL are exerted through platelet activating factor (PAF)-like lipids and lysophosphatidylcholine (LPC) [6,7]. Both agents are generated in the oxidation of the omnipresent phospholipid, phosphatidylcholine, which is abundant in LDL and plasma membranes [8,9]. This group of pro-inflammatory/cytotoxic compounds generated in the oxidation of LDL exhibits the phosphorylcholine (PC) epitope and PC is one of the key epitopes found on oxLDL but not native LDL [10,11]. It is noteworthy that, two out of three monoclonal antibodies (E06 and DLH3) commonly used in assays to quantify serum oxLDL levels, target the PC-moity [12]. With the pathological character of oxLDL and its related oxidized lipids in mind, beneficial effects of anti-PC antibodies have been reported, both in vitro [10,13–15] and in vivo [16–18].

Most humans have a substantial immune response to PC and natural PC-specific antibodies (anti-PC) have been reported to

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constitute between 5–10% of the total IgM pool [19]. The population of anti-PC antibodies in serum is generally subdivided into two idiotypes, Group I and II, based on their affinity for two haptens [20]. Group I antibodies binds both phosphorycholine (PC) and *p*-nitrophenyl phosphorylcholine (NPPC) whereas Group II antibodies require the phenyl group of NPPC in order to bind [20]. Although this distinction has been known for a long time, the differing roles of these two anti-PC idiotypes have not been studied in the context of health and disease.

We have had a long interest in studying anti-PC antibodies with focus on anti-inflammation in atherosclerosis. After developing a standardized protocol for measuring anti-PC IgM, we have analyzed serum samples from several large CVD-cohorts in Sweden. The findings have consistently been that low levels of anti-PC IgM are associated with CVD and that high levels are correlated with reduced rate of atherosclerosis progression [14,21–24].

The European Lacidipine Study on Atherosclerosis (ELSA) is a 4-year prospective study on hypertension treatment in relation to development of atherosclerosis in the carotid artery [25]. From this cohort, we have previously determined that high levels of anti-MDA-LDL, anti-OxLDL and anti-PC IgM (but not IgG) are protection factors for atherosclerosis development [21]. In the present study we explore the roles of anti-PC IgG1, IgG2 and IgA, different antibody idiotypes as well as the long term stability of anti-PC IgM levels. We have also studied combinations of anti-PC with anti-MDA-LDL or anti-oxLDL.

2. Methods

2.1. Subjects and acquisition of baseline data

Serum samples were obtained prior to enrollment and again at follow-up from 226 individuals with established hypertension (diastolic pressure >95 mm Hg) who participated in the Swedish component of the European Lacidipine Study on Atherosclerosis (ELSA). During the admission process, information on age, gender, blood pressure, weight, height, smoking habits and previous medical history were recorded along with laboratory values of different parameters including creatinine, fasting glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides. For detailed information on ELSA, please refer to the original article [25]. The study was approved by the Ethics Committee of the Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

2.2. Carotid ultrasound

The mean of the maximum Intima-Media Thicknesses (IMT) in the far walls of common carotids and bifurcations (CBM_{max}) was determined by B-mode ultrasonography at the time of inclusion, and 4 years afterwards. All scans were performed with the Biosound 200 II device (All Imaging Systems inc., Irvine CA, USA) and read at the Ultrasound Coordinating Center with quality assurance accomplished as reported. The levels of the different anti-PC antibody classes/subclasses at enrollment were evaluated with respect to increase or decrease in IMT at the 4-year follow up.

2.3. Purification of serum anti-PC IgG and IgM

Pooled human IgG (Baxter, Deerfield IL, USA) and IgM (Sigma Aldrich, St. Louis MO, USA) was passed over PC-Sepharose columns (Biosearch technologies, Novato CA, USA). The columns were then washed with Phosphate Buffered Saline (PBS) pH 7.4 with Tween20 to remove non-bound immunoglobulins. These non-anti-PC antibodies were collected to be used as control antibodies (flowthrough immunoglobulins) in later experiments. The bound PC-specific antibodies were then eluted with 0.01 M acetic acid and concentrated/buffer exchanged to PBS pH 7.4 using Centricon Plus-70 centrifugation filter units (Millipore, Billerica MA, USA).

2.4. Determination of antibody levels and binding specificities

Detection of IgM anti-PC antibodies was performed with an enzyme linked immunoassay (ELISA) using Athera CVDefine™ kit (Athera Biotechnologies AB, Stockholm, Sweden). The kit is based on PC covalently linked to bovine serum albumin (PC-BSA) coated onto 96-well Nunc Maxisorp micro-titer plates. The assay was carried out in accordance with the manufacturer's recommendations. All readings of results were performed on ELISA Multiscan Plus spectrophotometer (Molecular Devices Emax, San Francisco).

For the determinations of anti-PC IgA, IgG1 and IgG2 titers, we utilized the pre-coated CVDefine plate combined with isotype-specific secondary antibodies purchased from Sigma Aldrich (goat anti-human IgA) and Invitrogen (monoclonal mouse anti-human IgG1/IgG2).

The binding specificity of human anti-PC IgG1, IgG2, IgM and IgA were determined in a competitive ELISA with p-nitrophenyl-phosphorylcholine (NPPC) hapten or phosphorylcholine (PC) hapten in accordance with previously published work [20]. Briefly, hapten was mixed with pooled IgA or affinity purified anti-PC from pooled IgG/IgM and incubated on CVDefine plates. Antibody of each isotype was then detected with the above-mentioned class/subclass specific secondary antibodies.

Anti-oxLDL and anti-MDA-LDL were analyzed by ELISA as previously described [26]. In summary, LDL was isolated from plasma of healthy donors by sequential preparative ultra-centrifugation and oxidized using copper ions (oxLDL) or derivatized with MDA (MDA-LDL). These were then coated on microplates, which were later blocked with 20% adult bovine serum in PBS (20% ABS-PBS). Diluted serum samples were incubated overnight at 4 °C. The presence of specific antibodies in the serum was detected using goat anti-human IgG ALP/anti-human IgM ALP in combination with substraste (pNPP) and read at 405 nm.

2.5. Cell viability assay

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using the standard protocol of Ficoll density gradient centrifugation. The freshly produced PBMC were counted and resuspended in RPMI 1640 before being seeded into 24-well plates at a concentration of 3×10^6 cells per ml.

L-α-Lysophosphatidylcholine (LPC) from egg yolk (Sigma) was first dissolved in ethanol and then further diluted in RPMI 1640 to a working stock solution. LPC was added to the cells of each well, either by itself, together with purified anti-PC IgM, total IgM or flowthrough IgM. After a 18 hour incubation period, cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Live cells with functioning mitochondria metabolize MTT to formazin, which absorbs light at 570 nm. The viability of the cells in each well was thus quantified by collecting the insoluble formazin formed in each well, dissolving it in DMSO and reading the optical density (OD) at 570 nm.

2.6. Statistical analysis

Antibody levels were dichotomized at the 75th, 90th and 95th percentiles. The association between antibodies and the

progression of atherosclerosis over a 4-year period were determined by estimating increases in IMT (yes or no) using conditional logistic regression analysis. Adjustments were made for possible confounders including age, smoking habits, serum cholesterol, serum triglycerides and mode of anti-hypertensive treatment (lacidipine, atenolol). To distinguish the fine specificities of different anti-PC isotypes, we utilized the unpaired student *t*-test. These analyses were performed using SAS 9.2 release (SAS institute, Cary NC, USA). For all statistical analyses, a two-tailed p-value < 0.05 was considered significant.

3. Results

3.1. Baseline characteristics

The baseline characteristics of the participants at the time of admission into the study have been detailed elsewhere [25] and are presented in Table 1.

3.2. Serum levels of anti-PC IgM, IgG1 and IgA could predict IMT-progression

Measurable levels of anti-PC IgG1 and IgG2 were found in most subjects whilst levels of IgG3 and IgG4 were generally undetectable. Thus only anti-PC IgG1 and IgG2 were included in the subsequent investigation.

Table 1
Basic characteristics of the study group at enrollment. Results are presented as means (S.D.) BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein.

	Total (N=226)
Age (years)	57.7 (7.8)
Sex (% males)	50
BMI (kg/m^2)	26.7 (3.7)
Total cholesterol (mg/dl)	232.4 (37.8)
HDL (mg/dl)	55.6 (27.6)
LDL (mg/dl)	149.4 (37.8)
Triglycerides (mg/dl)	131.6 (58.2)

Table 2
Summary of results from regression analysis for each dichotomized antibody class in the prediction of changes in IMT over a 4-year period adjusting for smoking, gender, total cholesterol, plasma triglycerides, age and hypertensive treatment in subjects with established hypertension.

Variable	Odds ratio	95% CI		p
		Lower	Upper	
Above 75th percentile				
anti-PC IgM	0.64	0.33	1.22	0.17
anti-PC IgG (total)	0.60	0.32	1.1	0.10
anti-PC IgG1	0.40	0.21	0.76	0.005
anti-PC IgG2	0.88	0.47	1.63	0.77
Above 90th percentile				
anti-PC IgM	0.019	0.07	0.52	0.001
anti-PC IgG (total)	0.60	0.25	1.4	0.24
anti-PC IgG1	0.22	0.08	0.60	0.003
anti-PC IgG2	0.96	0.38	2.43	0.94
Above 95th percentile				
anti-PC IgM	0.05	0.006	0.40	0.006
anti-PC IgG (total)	0.72	0.21	2.44	0.59
anti-PC IgG1	0.24	0.06	0.97	0.045
anti-PC IgG2	0.76	0.22	2.63	0.66
Below 25th percentile				
anti-PC IgA	2.46	1.22	4.99	0.012

We found no correlation between anti-PC IgG2 and IMT-changes. However, a high level of the IgG1 subclass at baseline was strongly predictive of no increase in IMT after four years (Table 2).

Testing serum for anti-PC IgA showed that subjects with low levels of anti-PC IgA had increased risk for IMT-progression. A protective effect at high levels was not seen for anti-PC IgA (Table 2).

3.3. Fine specificity differences among the anti-PC isotypes

The fine specificity profiles of anti-PC IgM, IgA, IgG (total), IgG1 and IgG2 were determined in pooled fractions of immunoglobulins to minimize the impact of individual variation. Human anti-PC IgM (Fig. 1a) and IgA (not shown) were found to be exclusively Group I. The IgG fraction, however, was determined to contain both Group I and Group II antibodies. Detailed examination with subclass specific antibodies revealed a significant discrepancy between anti-PC IgG1 and IgG2 with regard to specificity.

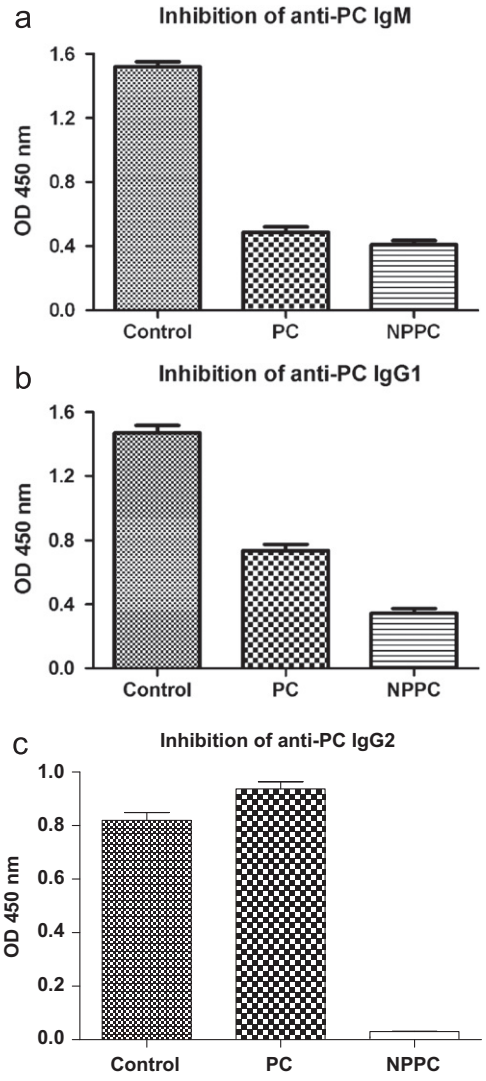

Fig. 1. The idiotypes of human anti-PC IgG1, IgG2 and IgM were determined in a competitive ELISA assay with p-nitrophenylphosphorylcholine (NPPC) hapten or phosphorylcholine (PC) hapten. The hapten concentration was 0.001 M and all experiments were performed in triplicate. (a) Anti-PC IgM has equal affinity for PC and NPPC. (b) Anti-PC IgG1 has affinity for both PC and NPPC. (c) Anti-PC IgG2 is only inhibited by NPPC and does not exhibit any affinity for PC.

Whereas the IgG1 pool is mostly Group I (Fig. 1b), the IgG2 pool is clearly made up of Group II anti-PC antibodies (Fig. 1c).

3.4. Combining anti-PC IgM with other autoantibodies against modified LDL improves the risk assessment

Combining data on anti-PC IgM (90th percentile) with anti-MDA-LDL (90th percentile) or anti-oxLDL IgM (90th percentile) strengthened the negative association with IMT-progression, Table 3. The combinations are stronger than each of the individual markers by themselves. However, just using the top 5th percentile of anti-PC IgM alone actually turns out to be an even better instrument for foreseeing IMT-changes, Table 2. Among the twelve study participants in the top 5th percentile, only one had IMT-progression after four years. For those below the 95th percentile, the incidence of IMT-progression was 137 cases in 214 subjects.

3.5. The serum levels of anti-PC IgM and IgG1 are strongly correlated

Levels of anti-PC IgG1 and IgM are highly correlated in individuals. Spearman's rank correlation coefficient (Srcc) between the two is 0.76 ($p < 0.0001$). Combining data on anti-PC IgM and IgG1 yields very little in increased predictive accuracy. The two variables are almost interchangeable and do not add any unique information to each other. Anti-PC IgM and IgA are also somewhat associated (Srcc = 0.36) in this group of individuals.

3.6. Anti-PC IgM inhibits LPC induced cytotoxicity

Both purified anti-PC IgM and total IgM was able to inhibit LPC-induced cytotoxicity ($p < 0.05$) in human immune cells (Fig. 2). The effect seen with the flowthrough IgM fraction (largely depleted of anti-PC IgM) was not significant.

Table 3
Combining anti-PC IgM (highest 10th percentile) with anti-MDA-LDL IgM or anti-oxLDL IgM improves the predictive accuracy of IMT-changes.

	Odds ratio	95% CI	*p*
aOxLDL IgM (highest 25th percentile)	0.12	0.03–0.42	0.0011
aMDALDL (highest 25th percentile)	0.14	0.04–0.52	0.0031
aOxLDL IgM (highest10th percentile)	0.12	0.03–0.54	0.0061
MDALDL (highest 10th percentile)	0.10	0.01–0.85	0.0347

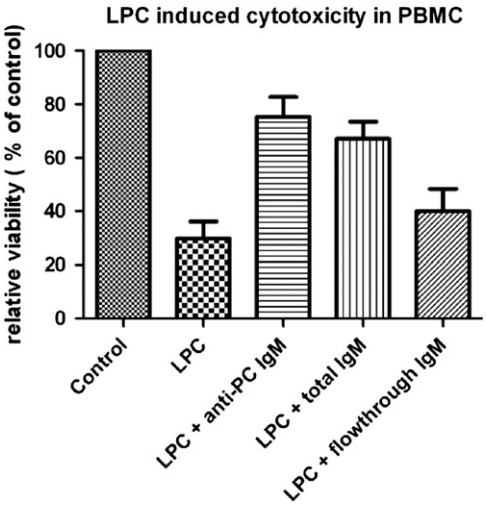

Fig. 2. LPC (16 μg/ml) was added to peripheral blood mononuclear cells. Either alone or together with antibodies (20 μg/ml). Anti-PC IgM and total IgM (which contains anti-PC IgM) inhibited LPC-induced cytotoxicity ($p < 0.05$) whereas flowthrough IgM, which is almost void of anti-PC does not significantly do so.

3.7. Serum levels of anti-PC IgM are stable over a four year period

The level of anti-PC IgM was measured in serum samples taken at two time points, four years apart (admission and follow-up). During this time period, levels of anti-PC IgM changed remarkably little in most patients. The Spearman rank correlation coefficient between the samples taken 4 years apart was determined to be 0.92 with a *p*-value of less than 0.0001.

4. Discussion

We here report that anti-PC IgM, IgA and IgG1 are associated with decreased likelihood of IMT-progression in hypertensive study subjects. This is not seen for anti-PC IgG2, which has a different affinity profile.

Anti-PC research started many decades ago when products from certain myeloma cell lines were identified as PC-reactive [27]. These antibodies were originally thought to be directed against certain pathogenic bacteria that display the PC-moity, including *Streptococcus pneumonie* [28]. However, an increasing body of evidence has since suggested a homeostatic function for anti-PC besides its role in infection defense [29]. It was reported in 2000 that oxLDL and apoptotic cells display the PC epitope [10] and later studies have shown that anti-PC antibodies can aid in the clearance of these pro-inflammatory agents and even prevent the formation of foam cells [14]. Our group recently published an article demonstrating that anti-PC extracted from human serum can inhibit the pro-inflammatory effect of PAF whose analogs formed through lipid oxidation are believed to be major inflammatory mediators in the atherosclerotic plaque [30]. It is possible that these natural antibodies, which make up a sizeable portion of the human antibody pool constitute a clearance system for aging and/or oxidized or otherwise modified lipoproteins and dying cells [31]. After all, it is generally known that anti-PC antibodies belong to a set of natural antibodies produced by $CD5^+$ B1 B-cells of the innate immune system independent of external antigens as demonstrated in germ free mice [10]. This "house-keeping" model maintains that high level of anti-PC is beneficial and insufficiency predisposes individuals for diseases related to chronic inflammation such as atherosclerosis [10].

Anti-PC antibodies are subdivided into two populations based on their affinity for phosphorylcholine (PC) and *p*-nitrophenyl-phosphorylcholine (NPPC) [20]. We have demonstrated that human anti-PC IgM, IgA and IgG1 are Group I anti-PC antibodies (similar to the murine T-15 clone), whereas anti-PC IgG2 is entirely made up of Group II antibodies. Fig. 1c shows that PC-hapten does not inhibit anti-PC IgG2, quite the contrary. Adding PC strengthens the signal from anti-PC IgG2, possibly by neutralizing Group I (IgG1) antibodies and thereby freeing up space on the ELISA plate for more anti-PC IgG2 to bind, enhancing its signal.

Patients suffering from periodontal diseases have an elevated risk for CVD [32] even though they develop high titers of anti-PC IgG2 [33]. This finding contradicts the "house-keeping" hypothesis, in which high levels of anti-PC antibodies are supposed to prevent atherosclerosis and CVD. However, our new data provides an explanation. We have clearly demonstrated that only antibodies of the Group I idiotype are associated with decreased atherosclerosis progression. The level of anti-PC IgG2 (Group II) was not associated with IMT-changes at all ($p = 0.94$). Given that the IgG2 antibodies in general are used to counter carbohydrate antigens [34], it is likely that anti-PC IgG2 is directed against capsulated bacteria. Human anti-PC IgG2 has, in fact, been implicated as a bactericidal protective factor against *Haemophilus influenza* and *Streptococcus pneumonie* [35].

Anti-PC IgG2 was found to be non-protective in this cohort. It is possible that this is related to the inability of the IgG2 subclass to engage Fc-receptors and recruit complement. However, the fact that anti-PC IgG2 has a different fine specificity and is heavily induced during infections suggests that this isotype of anti-PC might be primarily involved in infection defense. The serum level of anti-PC IgG2 is thus, likely determined through infections by PC-bearing pathogens and hence unrelated to CVD.

We have previously demonstrated that anti-PC can inhibit the formation of foam cells and neutralize the pro-inflammatory effect of PAF [14,30]. In this study, we have identified an additional mechanism through which anti-PC could confer protection against CVD and atherosclerosis, where dead cells are abundant. Apoptosis is known to weaken advanced atherosclerotic plaques [36] and inhibiting LPC-induced cell death could be very important in stabilizing plaques that might otherwise rupture, especially considering the richness of LPC in plaques [37].

Anti-PC IgM has consistently shown significant negative correlations with CVD [14,21–24]. This study introduces two new biomarkers, anti-PC IgG1 and IgA. Spearman rank correlation coefficients show that levels of anti-PC IgM, IgA and IgG1 are all associated. This implies that it is only necessary to measure one antibody class since measuring more classes would be redundant. Given that all previous publications have been about anti-PC IgM and the availability of a ready-to-use ELISA kit, it may be wise to use anti-PC IgM for risk assessments. However, the role of anti-PC IgA deserves further study due to its intricate connection with gut immunity.

We have previously shown that high levels of anti-PC IgM, anti-MDA-LDL and anti-oxLDL are negatively associated with IMT progression in this cohort [21]. In the present study, we have demonstrated that combining anti-PC IgM with anti-MDA-LDL or anti-oxLDL yields two new composite protective parameters which are superior to any of the three markers by themselves. Anti-MDA-LDL like anti-PC is a natural antibody that has been widely studied in the context of CVD [15]. The finding that the two antibodies can act in synergy opens up exciting avenues related to in vitro experiments as well as immunization strategies involving induction of both anti-PC and anti-MDA-LDL. Although combining the three different antibodies produces impressive odds ratios (OR), the best OR is obtained by looking at the very highest levels of anti-PC IgM alone (above the 95th percentile) where the OR is 0.05. While the limited number of individuals in this group urges caution, it is still a striking finding.

Long term stability is important quality for a biomarker that makes claim to predict atherosclerosis progression and cardiovascular events many years in the future. A previous study indicated that anti-PC IgM was constant over a period of many weeks [38]. Here we have, for the first time, shown that the levels are steady over a four year period.

In summary, the serum levels of Group I anti-PC antibodies can be used to predict progression of carotid IMT in patients with hypertension. Of the different Group I isotypes, particularly anti-PC IgM stands out as a stable biomarker candidate, which at very high levels is associated with a striking decrease in likelihood of atherosclerosis progression. One possible novel biological explanation for this observation is that anti-PC IgM can inhibit LPC-induced apoptosis and thus stabilize atherosclerotic plaques.

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Disclosures

JF and UdF are named as inventors on patent applications or granted patents relating to anti-PC.

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